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F. Couch

PI - Signature Date

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1999 Annual Report DAMD17-97-1-7048

Overview

The purpose of this award is to study the transcriptional regulation of the BRCA2 breast cancer predisposition gene with the goal of identifying agents capable of modulating BRCA2 expression. The most recent report for this grant was submitted on February 9th, 1999. The delay in submission resulted from the renegotiation and transfer of the grant to the Mayo Clinic and Foundation. Therefore, this report will describe the work that has been completed since February.

Experimental Results

Identification of the basic transcription regulatory region of BRCA2 promoter.
In the previous report the delineation of an approximately 900bp region of the BRCA2 promoter that is responsible for basal transcriptional regulation of BRCA2 in MCF7 and 293T cells was described. Further characterization of this region has been undertaken by testing transcription from deletion mutants of the promoter in a luciferase reported assay. Briefly, each BRCA2 experimental reporter construct was transfected into MCF-7 breast epithelial cell line with a construct of renilla luciferase (HSV-TK) gene, which was used as an internal control for normalizing the activity of the experimental reporter. The firefly and renilla luciferase activities were detected in the cell lysates after 48 hours post-transfection. Relative firefly luciferase activity was measured as the ratio of the activity of firefly luciferase to renilla luciferase. The transcription activity of different mutants of the 8 kb region are shown in Figure 1. The results of transfection of the mutants indicated that the BRCA2 promoter is regulated in a complex fashion. Several regions appear to contribute 2 to 3 fold activation or repression. Mild 3 fold repression is observed between -582 and -515. Activation (2 fold) is observed between +110 and +181, while a 3 fold effect is present upon deletion of the region between +110 and 0 (transcription start site), and between -58 and -144. While these changes are statistically significant and suggest the presence of activation and repression elements, it is also possible that alterations in the size of the promoter region in the luciferase construct may artifactually induce these effects. However, a 20 fold loss of luciferase activity was observed following deletion of a 40 bp region (-58 to -18), suggesting that this region contains cis-elements that are critical for regulation of basal transcriptional activity of the BRCA2 promoter. This work represents an extension of the research proposed in Tasks 1, 4, 5, and 6.

Further Characterization of the 44 base pair nucleotides.
The 40bp region will include any elements that overlap this region. Therefore, we extended the region to 44bp to include a number of overlapping sites including USF, c-myc, and CDE. The 44bp interval was predicted to contain binding sites for several transcription factors, such as c-myc, activating transcription factor (ATF), and upstream stimulatory factor (USF), and aryl hydrocarbon receptor which binds the xenobiotic-responsive element (XRE). A further series of deletions were constructed to more accurately map the actual controlling element. Deletion of 10 bp from -58 to -50 did not

affect activity. Deletion of 16 more nucleotides (from -49 to -35, including two XRE motifs) resulted in 2-fold loss of luciferase activity. Similar results were obtained by point mutation of XRE sites, indicating that XRE may be involved in BRCA2 transcription up-regulation. The sequence from -34 to -14 contained two repeating sequences (GCGTCACG) and overlapping consensus binding sites for ATF, c-myc, USF, and CDE (centromere DNA element). Substitution of the CGCGTCACG nucleotides (from -33 to -26) and / or the CGTCACGTG nucleotides from -24 to -16 by TACACTATT resulted in 10 to 100 fold loss of luciferase activity (Figure 2). This suggests that an element within the -33 to -26 sequence is required for transcription of the BRCA2 promoter, while an element located between -24 to -16 is an important contributor to promoter function. Given the repetitive nature of this region it is likely that homo or heterodimerization of transcription factors binding to these repeats controls BRCA2 transcription. This work in part addresses the research proposed in Tasks 6 and 7 of the SOW.

USF may be an important factor in the BRCA2 transcription regulation. In order to identify the specific cis-acting elements from the -33 to -14 region, and the specific transcription factors that bound to these elements we performed protein-DNA binding assays. Electrophoretic mobility shift assays were performed using oligonucleotide probes containing either wildtype sequence for the repeat region (-34 to -14) or mutations of either and both of the repeat sequences. Gel shift and competition assays of MCF7 lysates with these oligonucleotides detected two specific protein-DNA complexes. The larger complex was ablated when the first repeat sequence from -33 to -26 was substituted with TACACTATT (probe: M-1). The smaller complex was not detected when the wild type sequence (-24- to -14) was replaced by TACACTATT (probe: M-2). Furthermore, the smaller complex was super shifted by antibody against USF (kindly provided by Michele Sawadogo) but was not affected by antibodies against c-myc (Santa Cruz Biotechnology) or ATF2 (Santa Cruz Biotechnology). The protein-DNA binding experiments suggest that USF can specifically interact with its DNA binding motif in BRCA2 promoter in the -24 to -14 region. Thus, USF may be an important regulator of BRCA2 expression. However, the protein component of the larger complex which is necessary for BRCA2 transcription has not yet been identified. This work in part addresses the aims of Tasks 7 and 9 of the SOW.

Future Experiments

1) To identify the transcription factor that binds to the -34 to -26 element.
2) To reconstitute activation of the BRCA2 promoter by expression of USF and the unknown binding promoter
3) To assess the role of homo or heterodimerization of these proteins in activation of the BRCA2 promoter.
4) To identify regulatory elements in the other activating and repressing domains of the promoter.
5) To identify modulating agents that contribute to regulation of the promoter. While this work was proposed for months 2-14 (Tasks 2 and 3), it has been delayed while

we have concentrated on the experiments described above. This work will now be undertaken in conjunction with Task 8.

Figure 1: Identification of the basic transcription regulatory region of BRCA2 promoter

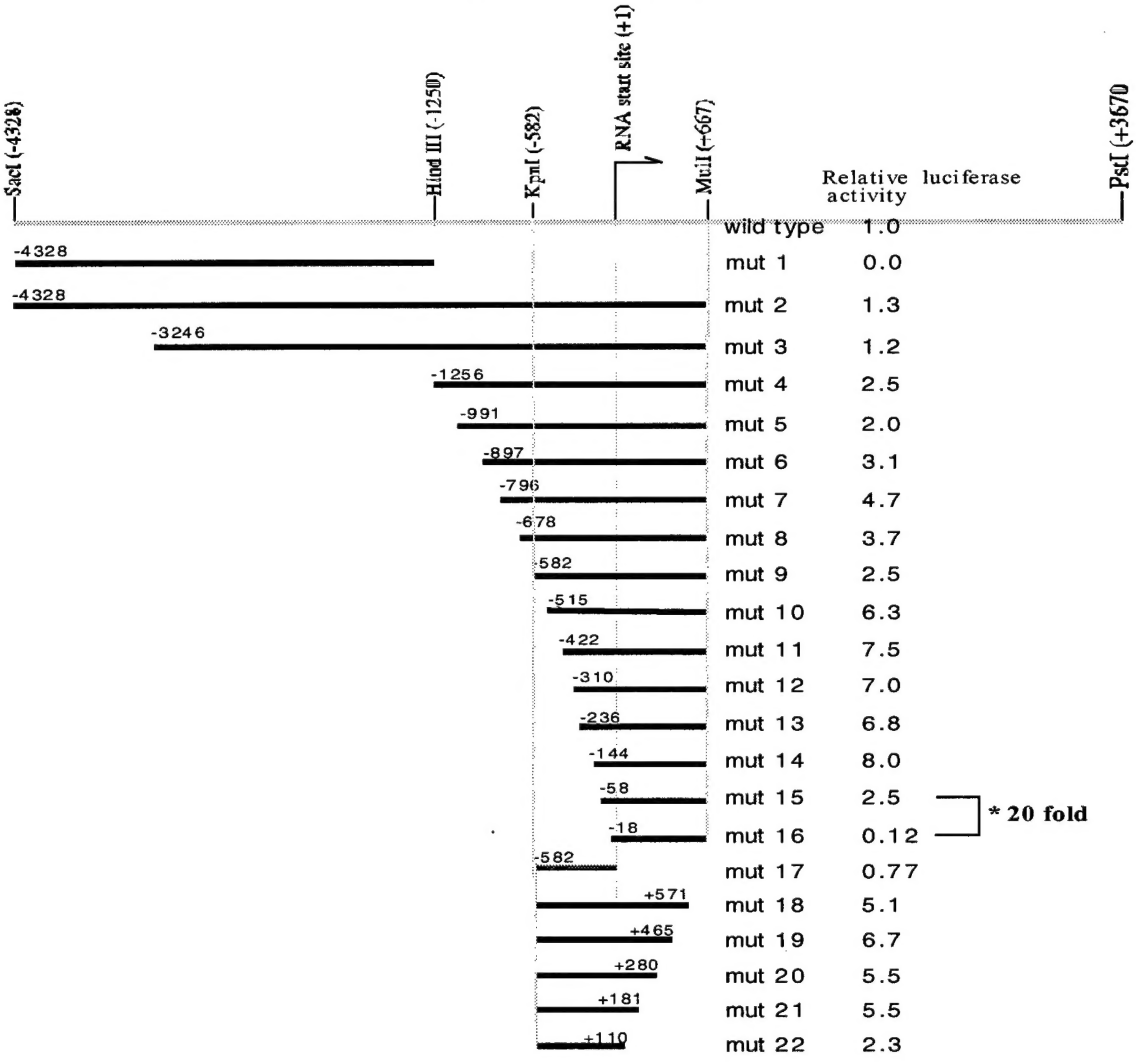

Figure 2: Mutiple elements were involved in the transcription regulation of BRCA2 within the 44 bp region